Medicinal Plant 2023, 14 (4): 38 – 40
DOI: 10.19600/j.cnki.issn2152 – 3924.2023.04.010

Determination of the Content of Five Active Components in Toad Skin

Xue JIANG[1], **Wenli LI**[2,3], **Yong CHEN**[2], **Jiabao MA**[1*]

1. The First Affiliated Hospital of Guangxi University of Chinese Medicine, Nanning 530023, China; 2. Guangxi University of Chinese Medicine, Nanning 530200, China; 3. The Second People's Hospital of Qinzhou, Qinzhou 535009, China

Abstract [**Objectives**] To establish a method for the determination of active components in toad skin. [**Methods**] HPLC method was used to determine the content of five active components (bufotalin, cinobufotalin, bufalin, cinobufagin and resibufogenin) in toad skin. [**Results**] Chromatographic conditions are as follows: Agilent ZORBAX SB-C_{18} chromatographic column was used; acetonitrile (A)-0.3% glacial acetic acid (B) gradient elution (0 – 15 min, 28% A – 54% A; 15 – 35 min, 54% A – 54% A) was conducted; the flow rate was 0.6 mL/min; the detection wavelength was 296 nm; the column temperature was 30 ℃; the sample size was 10 μL. Under the above conditions, the determination method of the five components can be established at one time. [**Conclusions**] The method was stable and reliable, and can provide experimental basis for the development and utilization of active ingredients in toad skin.

Key words Toad skin, High performance liquid chromatography, Determination of content

1 Introduction

Toad skin, the skin of *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider of Bufonidae, was first recorded in *Benjingfengyuan*[1]. Toad skin can clear heat and detoxify, induce diuresis for removing edema, and treat boils, scrofula, tumors, infantile bloating and chronic trachea[2]. As a kind of ethnic medicine, toad skin has been included in the list of Zhuang medicines, and is a commonly used characteristic medicine in Guangxi. It is used for the treatment of breast cancer[3], lung cancer[4], gastric cancer[5-6], esophageal cancer[7], *etc.* Fresh toad skin is mostly a drug for external use, and it must be processed before being taken orally. At present, raw toad skin, which has not been processed, is mostly used as traditional Chinese medicine in decoction pieces. Toad skin has complex chemical composition, wide pharmacological activity and a large number of products on the market, which has become the focus of research by more and more scholars. However, there are few studies on the content determination of various active components of toad skin. The pharmacopoeia also lists the determination of two components contained in relatively pure toad venom[8], and the identification and detection of various active components can provide a reference for the development and utilization of toad skin.

2 Materials

2.1 Instruments Main instruments included 1110Agilent high performance liquid chromatograph (Shimadzu Company), vacuum drying box (Shanghai – Yiheng Scientific Instrument Co., Ltd.), 2000T high speed multi-functional mill (Yongkang Bao'ou Hardware Products Co., LTD.), TNC-R8 rechargeable portable juicer (Yongkang Tonik Industry and Trade Co., Ltd.), HWS-26 electric thermostatic water bath (Shanghai Qixin Scientific Instrument Co., Ltd.), JJ500 electronic balance (Changshu Shuangjie Test Instrument Factory), KQ5200B ultrasonic cleaner (Kunming Ultrasonic Instrument Co., Ltd.), ME204/02 electronic balance (METTLER TOLEDO Instrument Shanghai Co., Ltd.), XSR205 1/100,000 analytical balance (METTLER TOLEDO Instruments Ltd.), DHG-9203A thermostatic air drying oven (Shanghai Qixin Scientific Instrument Co., Ltd.), and Mili-Q ultra-pure water machine (Guangxi Nanning Bomei Biotechnology Co., Ltd.).

2.2 Medicinal materials and reagents The toad skin used in the experiment was purchased from Guangxi Wanbaotang Pharmaceutical Co., Ltd. Its detailed information is as follows: its Latin scientific name is *Bufo bufo gargarizans* Cantor; medicinal part: skin; origin: Guangxi; batch number: 210206901. After being tested by Yang Zhengteng, chief pharmacist of the First Affiliated Hospital of Guangxi University of Chinese Medicine, the quality standard was in line with the 2011 edition of the *Quality Standard for Zhuang Medicines of Guangxi Zhuang Autonomous Region*.

The main reagents included acetonitrile (Fisher Company, USA), methanol (Fisher Company, USA), methanol (2018121207, Sichuan Xilong Science Co., Ltd.), glacial acetic acid (191006, Chengdu Colon Chemical Co., Ltd.), bufotalin reference product (PS010808, Chengdu Pusi Biotechnology Co., Ltd., purity: ≥98%), cinobufotalin (ST16980105, Shanghai Standard Technology Co, Ltd., purity: ≥ 98%), bufalin (ST04960120, Shanghai Standard Technology Co, Ltd., purity: ≥98%), cinobufagin (PS012070, Chengdu Pusi Biotechnology Co., Ltd., purity: > 98%), and resibufogenin (1110885-200102, China Institute of Food and Drug Control, purity: > 98%).

Received: May 5, 2023 Accepted: July 2, 2023

Supported by the Self-funded Research Project of Administration of Traditional Chinese Medicine of Guangxi Zhuang Autonomous Region (GXZYZ20210078); Key Research and Development Project of Guangxi Science and Technology Department (Guike AB19110003).

* Corresponding author. E-mail: 674632459@qq.com

3 Methods and results

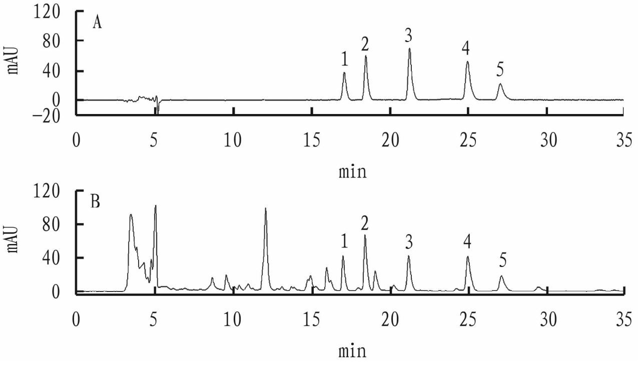
3.1 Conditions and analysis

3.2.1 Chromatographic conditions. Agilent ZORBAX SB-C_{18} chromatographic column was used. Acetonitrile (A)-0.3% glacial acetic acid (B) gradient elution (0 – 15 min, 28% A – 54% A; 15 – 35 min, 54% A – 54% A) was conducted; the flow rate was 0.6 mL/min; the detection wavelength was 296 nm; the column temperature was 30 ℃; the sample size was 10 μL.

3.2.2 Preparation of reference solution. At first, 5.58 mg of bufotalin, 9.00 mg of cinobufotalin, 11.00 mg of bufalin, 11.00 mg of cinobufagin and 2.62 mg of resibufogenin were weighed, and 0.558, 0.900, 1.100, 1.100, and 0.262 mg/mL control solutions were prepared by adding methanol to 10 mL volumetric bottle as reserve solutions. Afterwards, 2 mL of each of the above reserve solutions was taken into a 25 mL volumetric bottle, to which methanol was added until 25 mL, and finally 0.044 6, 0.072 0, 0.088 0, 0.088 0, and 0.021 0 mg/mL mixed reference solutions of bufotalin, cinobufotalin, bufalin, cinobufagin and resibufogenin were prepared.

3.2.3 Preparation of test product solution. Firstly, about 2 g of toad skin powder (passing through No. 5 sieve) was accurately weighed, and placed in a tapered bottle with a plug, to which 30 mL of methanol was accurately added. The solution was weighed, and extracted by reflux for 1 h. After being cooled, it was weighed again, and the lost weight was made up with methanol. Afterwards, it was shaken well and filtered. Finally, the filtrate was taken and centrifuged.

3.2.4 System adaptability test. Under the set chromatographic conditions, the separation of bufotalin, cinobufotalin, bufalin, cinobufagin and resibufogenin in the test product solution was good. The separation degree was greater than 1.5, and the theoretical plate number was not less than 5 000. The chromatograms are as shown in Fig. 1.



Note: A. Mixed reference solution; B. Test product solution. 1. Bufotalin; 2. Cinobufotalin; 3. Bufalin; 4. Cinobufotoxin; 5. Resibufogenin.

Fig. 1 Chromatograms

3.2.5 Investigation of linear relationship. Firstly, 0.5, 1, 2, 3, 4, and 5 mL of the mixed reference solutions in Section **3.2.2** was accurately absorbed into a 5 mL volumetric bottle, to which methanol was added until 5 mL. It was shaken well, and prepared into mixed standard solutions of different concentrations. The samples were injected according to the chromatographic conditions in Section **3.2.1**, and the peak area of bufotalin, cinobufotalin, bufalin, cinobufagin and resibufogenin was recorded. Standard curves were drawn respectively with the concentration of the control substance as the horizontal coordinate and the peak area as the vertical coordinate, and the regression equation was obtained by linear regression. The results showed that the five components had a good linear relationship within their respective concentration ranges (Table 1).

Table 1 Results of linear relationship

Reference substance	Regression equation	r	Linear range μg/mL
Bufotalin	$Y = 11.458X + 23.086$	0.999 3	4.46 – 44.6
Cinobufotalin	$Y = 11.158X + 8.404\ 7$	0.999 8	7.20 – 72.0
Bufalin	$Y = 11.658X + 24.951$	0.999 7	8.80 – 88.0
Cinobufotoxin	$Y = 11.051X + 26.310$	0.999 5	8.80 – 88.0
Resibufogenin	$Y = 22.606X + 16.054$	0.999 4	2.10 – 21.0

3.2.6 Precision test. At first, 10 μL of the mixed reference solutions in Section **3.2.2** was taken accurately, and the samples were injected continuously six times according to the chromatographic conditions in Section **3.2.1**, and the peak area of bufotalin, cinobufotalin, bufalin, cinobufagin and resibufogenin was recorded. *RSD* values were calculated, namely 2.51%, 1.96%, 1.88%, 1.06% and 1.81%, respectively, indicating that the precision of the instrument was good.

3.2.7 Stability test. The test solutions of toad skin in Section **3.2.3** were accurately taken, and samples were injected at 1, 2, 4, 6, 12, 18 h according to the chromatographic conditions in Section **3.2.1**, respectively. The chromatographic peak area of bufotalin, cinobufotalin, bufalin, cinobufagin and resibufogenin was recorded. *RSD* values were calculated, namely 1.99%, 2.87%, 2.33%, 1.95% and 2.63%, respectively, showing that the tested solution had good stability within 18 h.

3.2.8 Repeatability test. 6 samples of toad skin from the same batch were accurately weighed, and the test solutions were prepared according to the preparation method of test products in Section **3.2.3**. Each test product was injected according to the chromatographic conditions in Section **3.2.1**, and the chromatographic peak area of bufotalin, cinobufotalin, bufalin, cinobufagin and resibufogenin was recorded. Their contents and *RSD* values were calculated. *RSD* values were 1.80%, 2.06%, 1.11%, 2.33% and 1.69%, respectively, indicating that the method had good reproducibility.

3.2.9 Sample recovery test. At first, 6 samples of toad skin with known content were accurately weighed, and each was about 1 g. A single component reference solution equal to each component in the samples was accurately added to them to prepare the test solutions according to the preparation method in Section **3.2.3**, and the samples were injected according to the chromatographic conditions in Section **3.2.1**. The chromatographic peak area of bufotalin, cinobufotalin, bufalin, cinobufagin and resibufogenin was recorded. Their recovery and *RSD* values were calculated, and *RSD* values were

2.70%, 1.97%, 2.37%, 2.51% and 1.96%, respectively.

3.2 Content of five active components According to the test method in Section **3.1**, the content of 5 active components in toad skin was obtained (Table 2).

Table 2 Content of 5 active components in toad skin mg/g

No.	Bufotalin	Cinobufotalin	Bufalin	Cinobufotoxin	Resibufogenin	Total content
1	0.776 9	1.265 9	1.078 1	1.370 9	0.346 9	4.838 7
2	0.735 6	1.286 6	1.101 3	1.396 0	0.337 7	4.857 2
3	0.771 4	1.296 0	1.090 2	1.331 1	0.351 4	4.840 1
Average	0.761 3	1.282 8	1.089 9	1.366 0	0.345 3	4.845 3
RSD//%	2.940 0	1.200 0	1.060 0	2.400 0	2.020 0	0.220 0

4 Discussion

When the method for determining the content of active components in toad skin was established, three mobile phases, acetonitrile-0.5% potassium dihydrogen phosphate, acetonitrile-water, and acetonitrile-0.3% glacial acetic acid, were compared, and acetonitrile-0.3% glacial acetic acid was finally selected. Under the chromatographic conditions of the characteristics of toad venom in the 2020 edition of the Chinese Pharmacopoeia. The baseline was stable, and the separation degree of the target peak was high. Although the same effect was achieved with 0.5% potassium dihydrogen phosphate, the buffer salt was difficult to prepare, and it is easy to damage the chromatographic column and liquid chromatograph. The test products were prepared based on the second volume of the 2011 edition of the *Quality Standard of Zhuang Medicines of Guangxi Zhuang Autonomous Region*[9], and the method was stable and feasible.

References

[1] ZHANG L. The origin of the Benjing[M]. Beijing: China Press of Traditional Chinese Medicine, 1996. (in Chinese).

[2] Jiangsu New Medical College. Dictionary of traditional Chinese medicine [M]. Shanghai: Shanghai Science and Technology Press, 1986. (in Chinese).

[3] LIU X, WANG Y. Preliminary study on the anti-breast cancer effects and mechanisms of the aqueous extract of toad skin and its peptides[J]. Chinese Journal of Pharmacology and Toxicology, 2019, 33(9): 712. (in Chinese).

[4] XUE P, LI LL, XU PP, *et al.* Application of dried toad skin in the treatment of small cell lung cancer[J]. Chinese Journal of Traditional Chinese Medicine, 2019, 37(6): 1356–1358. (in Chinese).

[5] GAO DD, ZHANG J, LI H. Effects of Chanpi Ezhu decoction on adjuvant chemotherapy in the treatment of gastric cancer[J]. World Journal of Integrative Chinese and Western Medicine, 2021, 16(12): 2329–2333. (in Chinese).

[6] XU KY, WANG J, ZHOU YD. Toad skin, sal-ammonte and panax notoginseng in the treatment of gastric cancer: Three prescription by professor TONG Xiaolin[J]. Jilin Journal of Chinese Medicine, 2021, 40(8): 986–988. (in Chinese).

[7] TIAN L, LUO Y, YAN YB, *et al.* Effect of bufalin on cellular proliferation and apoptosis in human esophageal squamous carcinoma EC9706 cells [J]. Acta Academiae Medicinae Sinicae, 2012, 34(6): 556–562. (in Chinese).

[8] National Pharmacopoeia Commission. Pharmacopoeia of the people's republic of China: Part I[M]. Beijing: China Pharmaceutical Science and Technology Press, 2020. (in Chinese).

[9] Guangxi Zhuang Autonomous Region Food and Drug Administration. Quality standards of Zhuang medicines of Guangxi Zhuang Autonomous Region[M]. Guangxi: Guangxi Science and Technology Press, 2011. (in Chinese).